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ORIGINAL ARTICLE

Design, synthesis and antifungal evaluation of novel benzimidazole tertiary amine type of fluconazole analogues

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-demethylase;
Microwave irradiation;
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Abstract A novel series of compounds containing tertiary amine moiety, substituted benzimidazole and triazole ring, initial design by molecular docking study of this scaffold at the active site of the fungal enzymes lanosterol 14α-demethylase (homology modeled of *C. albicans*) was synthesized by microwave irradiation and characterized by Proton Nuclear Magnetic Resonance (^{1}H NMR), Infra Red (IR), and Mass Spectroscopy (MS), and by elemental analysis. The screening of compound for *in vitro* (turbidimetric method) and *in vivo* (kidney burden test) antifungal activity against *C. albicans* revealed activity in many of the compounds as comparable to that of fluconazole.

1. Introduction

In the past few decades, the incidence of life-threatening fungal infections caused by pathogenic fungi is becoming alarming. This scenario is being observed among individuals with suppressed immune systems brought about by the use of cytotoxic drugs, immunosuppressive therapy, or human immunodeficiency virus infection (Ting and Walker, 2008). These infections have also been observed in some iatrogenic or nosocomial clinical settings. Autopsy data indicate that more than half of the patients who die with malignancies are infected with *Candida* spp., approximately one-third with *Aspergillus* spp., and increasing numbers with *Cryptococcus* spp. or other fungi such as *Fusarium* spp. The major opportunistic pathogen has been *C. albicans* (Pfaller and Diekema, 2004). Although several superior antifungal drugs are available for the treatment of these life threatening infections they still demonstrate drawbacks such as water solubility, narrow spectrum and serious toxic side effects such as pruritis, gastrointestinal upset, thrombophlebitis, drug interference, bioavailability, and hepatotoxicity (Hester et al., 2004).

The major thrust of medicinal chemist in the area of antifungal chemotherapy for the last two decades has been focused on a group of heterocyclic compound generally referred to as the 'azoles'. Conventional azoles including chlormidazole

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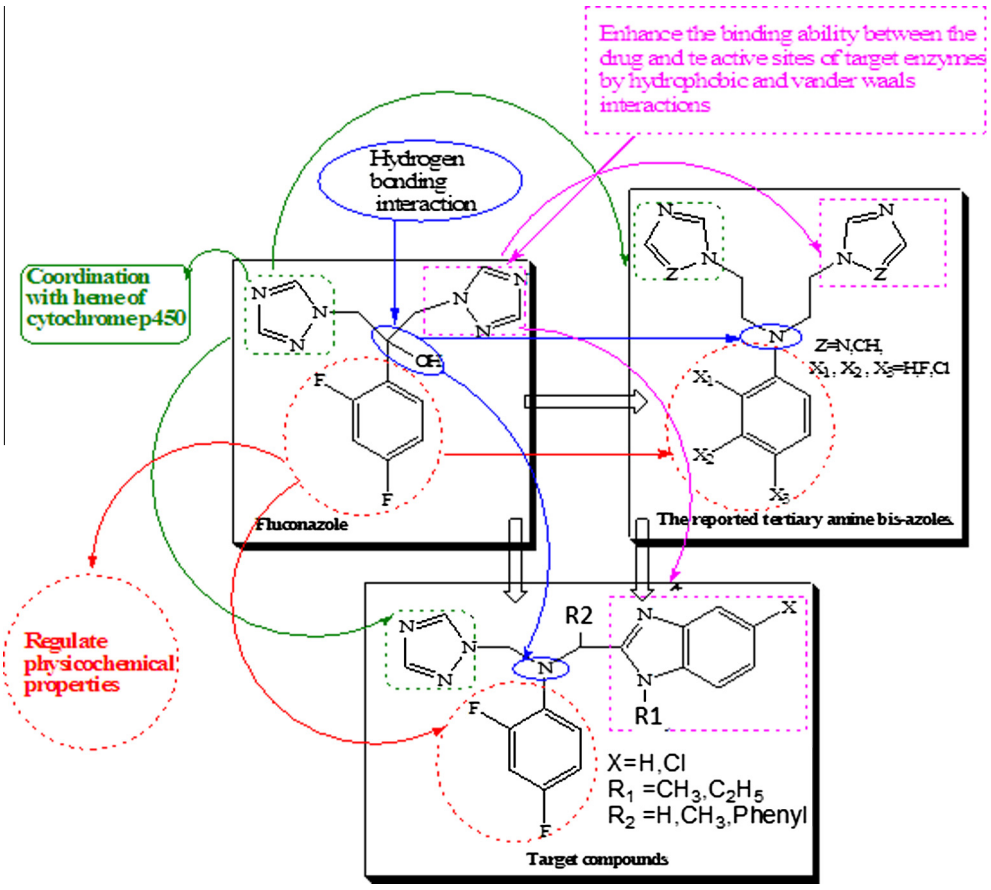

Figure 1 Design of benzimidazole tertiary amine type of fluconazole analogues.

(Erik et al., 1967), miconazole (Jiang et al., 2005), clotrimazole (Frye and Robinson, 1988), and ketoconazole (Brown et al., 1976) were highly substituted imidazoles. However, the structure activity studies have shown that 1,2,4-triazole ring (fluconazole, voriconazole, and albaconazole) enhances the selectivity for binding with lanosterol 14α-demethylase without adversely affecting the antifungal properties of the molecule (Groll and Lumb, 2012). Azole antifungal drugs show their action by the inhibition of lanosterol 14α-demethylase in the process of biosynthesis of ergosterol through a mechanism in which the heterocyclic nitrogen atom (N-4 of triazole) binds to the heme iron atom (Sheehan and Hitchcock, 1999).

However, the increasing administration of antifungal drugs has led to the development of resistance to these drugs. A survey by Pfaller et al. revealed that genetic mutations are possibly responsible for the resistance to clinically used drugs especially fluconazole (Pfaller et al., 2001). In addition, resistance to new structurally related azoles such as voriconazole (Jeu et al., 2003), and ravuconazole (Lepesheva et al., 2006) has also been observed in other studies. A perusal of literature revealed that tertiary amine moiety, benzimidazole and triazole rings were significant structural moieties for antifungal activity (Ates-Alagoz et al., 2005; Hernández-Luis et al., 2010; Özkay et al., 2010; Pawar et al., 2004; Jain et al., 2014; Mavrova et al., 2007; Garuti et al., 2000; Le et al., 2004; Thakurdesai et al., 2007; Ram et al., 1992; Abdel-monem Abdel-hafez, 2007; Güllerman et al., 2001; Maxwell et al., 1984; Sangshetti et al., 2011; Zhang et al., 2013; Wang et al., 2012). In view of the previous evidence available for bioactive benzimidazoles, the present study aimed to redesign the structure of fluconazole in several aspects as illustrated in Fig. 1.

Benzimidazole (imidazole ring fused to the benzene ring with a large conjugated system and a superior electro rich properties in comparison with triazole and imidazole) moiety, a bioisosteres was selected to replace triazole of fluconazole to evaluate the effect of substituted N1 and C2 benzene fused

Table 1 Ramachandran plot values showing number of residue in the favoured allowed, outlier region through RAMPAGE evaluation server.

Structure	Number of residues in favoured region (%)	Number of residues in allowed region (%)	Number of residues in outlier region (%)
4K0F	98	2	0
MODEL BUILT	96.6	3.0	0.4

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Table 2 Molecular docking data of synthesized compounds compared with fluconazole.

Compound code	Lig score 1	Lig score 2	-PLP1	-PLP2	Jain	PMF	Dock score	Distance of N_4 from heme iron (Å)
10a	2.81	2.97	58.54	64.36	1.3	52.72	32.622	3.009
10b	3.02	4.12	56.12	54.24	1.55	49.27	44.843	3.140
10c	3.69	5.51	79.96	86.31	2.8	51.14	32.789	3.226
10d	3.2	3.96	76.84	75.18	3.32	72.14	33.184	2.166
10e	1.79	1.81	52.73	50.28	4.13	46.35	43.97	3.198
10f	3.51	3.6	83.14	83.77	3.1	54.65	31.419	2.670
10g	1.74	1.69	87.76	100.88	5.34	65.35	22.154	3.010
10h	1.42	1.44	48.57	43.52	3.58	58.21	37.079	3.093
10i	5.11	5.65	88.53	82.15	2.7	68.64	51.448	2.243
10j	2.9	3.25	60.81	65.10	3.12	62.14	36.25	2.080
10k	1.96	2.52	54.32	49.24	3.96	44.58	41.90	3.159
10l	4.49	3.65	80.12	79.25	3.72	58.66	42.16	2.510
Fluconazole	5.17	5.19	73.88	65.13	1.19	62.97	54.846	2.392

Table 3 Effect of synthesized compounds against *C. albicans* by serial dilution method.

Compounds	Concentration (μmol/ml)						
	0.5	0.25	0.125	0.06	0.03	0.015	0.0076
10a	–	+	+	+	+	+	+
10b	–	–	–	–	+	+	+
10c	–	–	–	–	+	+	+
10d	–	–	–	+	+	+	+
10e	–	–	–	–	+	+	+
10f	–	–	–	–	+	+	+
10g	–	–	–	–	–	+	+
10h	–	–	–	–	–	–	+
10i	–	–	–	–	–	–	–
10j	–	–	–	–	–	+	+
10k	–	–	–	–	–	–	+
10l	–	–	–	–	–	–	+
Fluconazole	–	–	–	–	–	–	–

(–) Indicates absence of growth; (+) Indicates presence of growth;

imidazole ring on antifungal activity (Luo et al., 2009; Nowakowska et al., 2008). Benzimidazole ring could readily interact with active target in biological system via diverse non-covalent interactions such as hydrogen bond, π–π stacking, and hydrophobic effect as well as Vander Waals force. Addition of functional groups to the benzimidazole ring has resulted in interaction with extra binding region in binding site (Khalafi-Nezhad et al., 2005).

In the present study, we evaluated the effect of benzimidazole by substitutions at N-1, C2 and C5 positions. The following variations in the benzimidazole ring were evaluated in this study:

- Methyl substitution at N1.
- C5 is normally substituted with hydrogen/hydroxyl/chlorine.
- Substituent at C2 methylene/α-substituted ethyl/benzyl.

The compound with best binding interaction does not necessarily imply to exhibit the best drug activity in medicine. Additionally, the drug has to pass through various barriers to reach the target site within the body. In order to achieve the best activity, we replaced tertiary alcohol of fluconazole with bioisosteres tertiary amino moiety. The tertiary amino group in comparison with the tertiary alcohol group was able to not only form hydrogen bonds but also accept protons or form quaternary salts which result in enhanced water solubility (Khalafi-Nezhad et al., 2005).

Several studies from the past have demonstrated that incorporating halobenzyl moiety into organic molecules could greatly improve the pharmacological properties (Guven et al., 2007; Türkmen et al., 2011; Karegoudar et al., 2008). An enhanced pharmacological action results from an increased rate of absorption, transport of drugs *in vivo,* and a higher lipid solubility (Lin et al., 2005). The greater flexibility of the benzyl moiety as compared to phenyl group may show improved molecular biological properties.

The methylene bridge between tertiary alcohol group and triazolyl moiety in fluconazole was replaced by ethylene/benzyl to increase molecular flexibility. This substitution may show

Table 4A *In-vivo* anti fungal activity of synthesized compounds (Mortality in hours).

Group	Treatment	Mortality (*x*/5)					
		1 h	2 h	4 h	6 h	12 h	24 h
1	Vehicle	0/5	0/5	0/5	1/5	2/5	2/5
2	Standard (0.42 mg/kg)	0/5	0/5	0/5	0/5	0/5	0/5
3	10a-0.26 mg/kg	0/5	0/5	0/5	0/5	0/5	0/5
5	10a-0.42 mg/kg	0/5	0/5	0/5	0/5	0/5	0/5
5	10b-0.26 mg/kg	0/5	0/5	0/5	0/5	0/5	1/5
6	10b-0.42 mg/kg	0/5	0/5	0/5	0/5	0/5	0/5
7	10c-0.26 mg/kg	0/5	0/5	0/5	0/5	1/5	1/5
8	10c-0.42 mg/kg	0/5	0/5	0/5	0/5	0/5	1/5
9	10d-0.26 mg/kg	0/5	0/5	0/5	0/5	1/5	1/5
10	10d-0.42 mg/kg	0/5	0/5	0/5	0/5	0/5	1/5
11	10e-0.26 mg/kg	0/5	0/5	0/5	0/5	0/5	0/5
12	10e-0.42 mg/kg	0/5	0/5	0/5	0/5	0/5	0/5
13	10f-0.26 mg/kg	0/5	0/5	0/5	1/5	2/5	2/5
14	10f-0.42 mg/kg	0/5	0/5	0/5	0/5	1/5	1/5
15	10g-0.26 mg/kg	0/5	0/5	0/5	0/5	0/5	1/5
16	10g-0.42 mg/kg	0/5	0/5	0/5	0/5	0/5	0/5
17	10h-0.26 mg/kg	0/5	0/5	0/5	0/5	1/5	1/5
18	10h-0.42 mg/kg	0/5	0/5	0/5	0/5	0/5	1/5
19	10i-0.26 mg/kg	0/5	0/5	0/5	0/5	1/5	1/5
20	10i-0.42 mg/kg	0/5	0/5	0/5	0/5	0/5	1/5

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Table 4B *In-vivo* anti fungal activity of synthesized compounds (Mortality in days).

Group	Treatment	Mortality (x/5)			
		Day 0	Day 7	Day 14	Day 21
1	Vehicle	0/5	3/5	5/5	5/5
2	Standard (0.42 mg/kg)	0/5	0/5	0/5	0/5
3	10a-0.26 mg/kg	0/5	1/5	2/5	3/5
5	10a-0.42 mg/kg	0/5	1/5	2/5	2/5
5	10b-0.26 mg/kg	0/5	1/5	2/5	3/5
6	10b-0.42 mg/kg	0/5	0/5	1/5	2/5
7	10c-0.26 mg/kg	0/5	1/5	1/5	2/5
8	10c-0.42 mg/kg	0/5	0/5	1/5	1/5
9	10d-0.26 mg/kg	0/5	1/5	2/5	3/5
10	10d-0.42 mg/kg	0/5	0/5	1/5	2/5
11	10e-0.26 mg/kg	0/5	1/5	2/5	3/5
12	10e-0.42 mg/kg	0/5	0/5	1/5	2/5
13	10f-0.26 mg/kg	0/5	1/5	2/5	2/5
14	10f-0.42 mg/kg	0/5	0/5	1/5	1/5
15	10g-0.26 mg/kg	0/5	0/5	2/5	3/5
16	10g-0.42 mg/kg	0/5	0/5	1/5	2/5
17	10h-0.26 mg/kg	0/5	0/5	1/5	1/5
18	10h-0.42 mg/kg	0/5	0/5	0/5	1/5
19	10i-0.26 mg/kg	0/5	0/5	0/5	0/5
20	10i-0.42 mg/kg	0/5	0/5	0/5	0/5
21	10j-0.26 mg/kg	0/5	1/5	1/5	2/5
22	10j-0.42 mg/kg	0/5	0/5	0/5	1/5
23	10k-0.26 mg/kg	0/5	0/5	1/5	1/5
24	10k-0.42 mg/kg	0/5	0/5	0/5	1/5
25	10l-0.26 mg/kg	0/5	0/5	1/5	2/5
26	10l-0.42 mg/kg	0/5	0/5	1/5	1/5

Table 4C *In-vivo* anti fungal activity of synthesized compounds (Kidney Burden Test).

Group	Treatment	Log10 CFU/gm wet tissue
1	Vehicle	4.12 ± 0.036
2	Standard (0.42 mg/kg)	2.17 ± 0.122*
3	10a-0.26 mg/kg	3.98 ± 0.018*
5	10a-0.42 mg/kg	3.14 ± 0.086*
5	10b-0.26 mg/kg	3.78 ± 0.013*
6	10b-0.42 mg/kg	3.02 ± 0.027*
7	10c-0.26 mg/kg	3.08 ± 0.029*
8	10c-0.42 mg/kg	2.84 ± 0.086*
9	10d-0.26 mg/kg	3.42 ± 0.014*
10	10d-0.42 mg/kg	2.93 ± 0.017*
11	10e-0.26 mg/kg	3.82 ± 0.014*
12	10e-0.42 mg/kg	3.48 ± 0.013*
13	10f-0.26 mg/kg	3.04 ± 0.086*
14	10f-0.42 mg/kg	3.28 ± 0.013*
15	10g-0.26 mg/kg	3.83 ± 0.027*
16	10g-0.42 mg/kg	3.49 ± 0.016*
17	10h-0.26 mg/kg	2.96 ± 0.016*
18	10h-0.42 mg/kg	2.65 ± 0.029*
19	10i-0.26 mg/kg	2.60 ± 0.020*
20	10i-0.42 mg/kg	2.43 ± 0.049*
21	10j-0.26 mg/kg	3.55 ± 0.032*
22	10j-0.42 mg/kg	3.02 ± 0.051*
23	10k-0.26 mg/kg	2.92 ± 0.033*
24	10k-0.42 mg/kg	2.68 ± 0.045*
25	10l-0.26 mg/kg	2.92 ± 0.027*
26	10l-0.42 mg/kg	2.71 ± 0.036*

Values are expressed as MEAN ± SD at $n = 5$, One way Anova followed by Bonferroni test.
* $P < 0.001$ (significant) compared to the vehicle.

positive effect on the molecular binding ability to the microbial target (Fang et al., 2010).

In view of above consideration and based on molecular docking studies, we designed and synthesized a series of novel tertiary amine type of substituted benzimidazole derivatives as fluconazole analogues using microwave irradiation. The newly prepared derivatives were docked into the active site of homology modeled CYP51 of *C. albicans*, using Accelrys, Discovery Studio 2.1 Software, Inc., San Diego, CA. The chemical structures of the new derivatives were confirmed by elemental and spectral (^{1}H NMR and Mass) analyses. All compounds were investigated for *in vitro* and *in vivo* antifungal activities against *C. albicans*.

2. Materials and methods

All materials were procured from Sigma Aldrich and Merck specialties Pvt. Ltd. (Mumbai, India). Solvents were dried and distilled being used. Anhydrous sodium sulfate was used to dry the solvents. Syntheses were performed in a CEM Discover® monomode reactor and temperature was monitored by a built-in infrared sensor. Thin layer chromatography (TLC) analyses were carried out on aluminum plates (Merck) precoated with silica gel 60 F254 (0.2 mm), and spots were visualized with UV light and I_2. Liquid intermediates were checked for purity using gas chromatography (Pack column SE-30, OV-101, and capillary column BP-5). Gravity column chromatography was performed using silica gel (Merck 60). Melting points were taken in open glass capillary using Elico melting point apparatus and were uncorrected. Infra Red (IR) spectra were recorded on KBr pellets on a Shimadzu 1000 FTIR spectrometer in the range of 4000–200 cm^{-1}, Resolution 2.0 with number of scan – 45. Apodization; Happ-Genzel. Proton (^{1}H) Nuclear Magnetic Resonance (NMR) spectra of compounds were recorded on Bruker Advance II 400 NMR Spectrophotometer using $CDCl_3$ solvent, at SAIF, Punjab University, Chandigarh. Mass spectra of compounds were recorded on API 4000 Q TRAP LC/MS/MS system using electron spray ionization positive ion mass spectrometric technique, at NHRDF, Chitegaon, Nashik. Elemental analyses were performed on a Perkin-Elmer 2400 Analyser and are within ±0.4% of theoretical values.

2.1. Synthesis of tertiary amine type of substituted benzimidazole derivatives (Scheme 1)

*2.1.1. Synthesis of 4-substituted (H/Cl) N1-methyl/ethyl-2-nitroaniline (**2a–2d**)*

It was synthesized by using 4-substituted (H/Cl) 2-fluronitrobenzene **1a**, **1b** 25 ml (33.5 g, 0.24 mol) in dimethyl formamide as solvent and (0.804 mol) anhydrous potassium carbonate, (2.5 mol, 40% solution) of methylamine/ethylamine was slowly added it through dropping funnel in cold condition. After complete addition of methylamine/ethylamine solution the reaction mixture was kept in microwave for 10 min. The completion of reaction was checked by monitoring TLC. After completion, the reaction mixture was poured into ice cold water with stirring and extracted with the product with ether, the ether layer was separated and dried with sodium sulfate

Figure 2 Shows alignment between query and template sequence, identity is 55.9% and similarity 70.2%.

and then ether was removed by distillation to get products (Willitzer et al., 1978).

*2.1.2. Synthesis of 4-substituted N1-methyl/ethyl-o-Phenylenediamine (**3a–d**)*

4-substituted N1-methyl/ethyl-2-nitroaniline (2a, 2b) (5 mM), zinc dust (0.25 g) and ammonium chloride (10 mM) in 5 ml water were mixed thoroughly in a small beaker (25 ml). The reaction solid mixture was placed in a microwave oven (300 W) at 50% power level for 8–15 min, the progress of the reaction was monitored by checking the solubility of the solid reaction mixture product in dilute HCl, and when all the organic solid dissolved it was filtered to separate zinc dust, and neutralized with aqueous solution. The solid was separated by filtration and recrystallized from an appropriate solvent (aqueous ethanol) (Abdullah and Suliman, 2011).

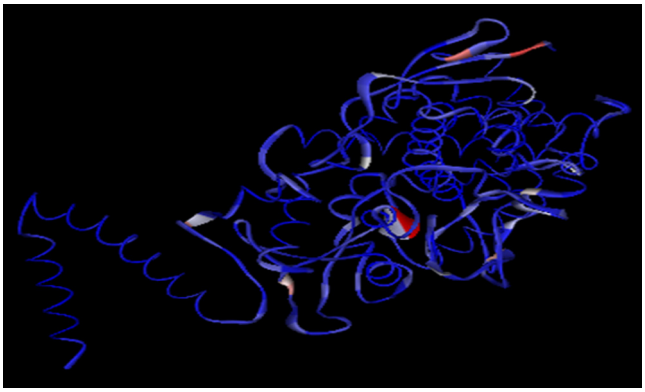

Figure 3 Homology modeled structure of lanosterol 14-alpha demethylase of *Candida albicans*.

*2.1.3. Synthesis of 5-substituted 2-(chloromethyl)-1-methyl-1H-benzo[d] imidazoles (**4a–d**)*

In a typical experiment, (0.2 mole) **3a–d**, (0.3 mole) of monochloro acetic acid, 5 ml of hydrochloric acid and 25 ml of methanol were taken in a round bottom flask and then placed in microwave irradiation at 250 W for 12 min. The reaction was monitored by TLC. A test portion was added in water and basified with ammonia solution. The solid was extracted with ether and TLC of this ether extract was done to check for completion of reaction. After completion, the reaction mixture was poured into ice-cold water. It was then basified with concentrated ammonia solution. The solid precipitate was filtered immediately and dried.

*2.1.4. Synthesis of 5-substituted- 2-(α-hydroxyethyl/phenyl)-1-methyl-1H-benzo[d] imidazoles (**5a–h**)*

4-substituted N1-methyl-o-Phenylenediamine 0.036 mol **(3a–d)**, lactic/mandelic acid 0.054 mol, 5 ml of hydrochloric acid and methanol were taken in a round bottom flask and then placed in microwave irradiation at 300 W for 15 min. The reaction was monitored by TLC. Reaction completion was monitored by TLC. Solution was allowed to stand overnight, filtered and the filtrate was cooled by addition of ice. It was then neutralized by careful addition of solid $NaHCO_3$ by stirring. Product removed by filtration, washed with water and dried. Recrystallization was done using chloroform by addition of charcoal.

*2.1.5. Synthesis of 5-substituted- 2-(1-chloroethyl/phenyl)-1-methyl-1H-benzo[d] imidazoles (**6a–h**)*

The activation of carboxylic function was carried out by using an excess of thionyl chloride and catalytic amount of dimethyl formamide placed in an ice cold water bath. To this mixture,

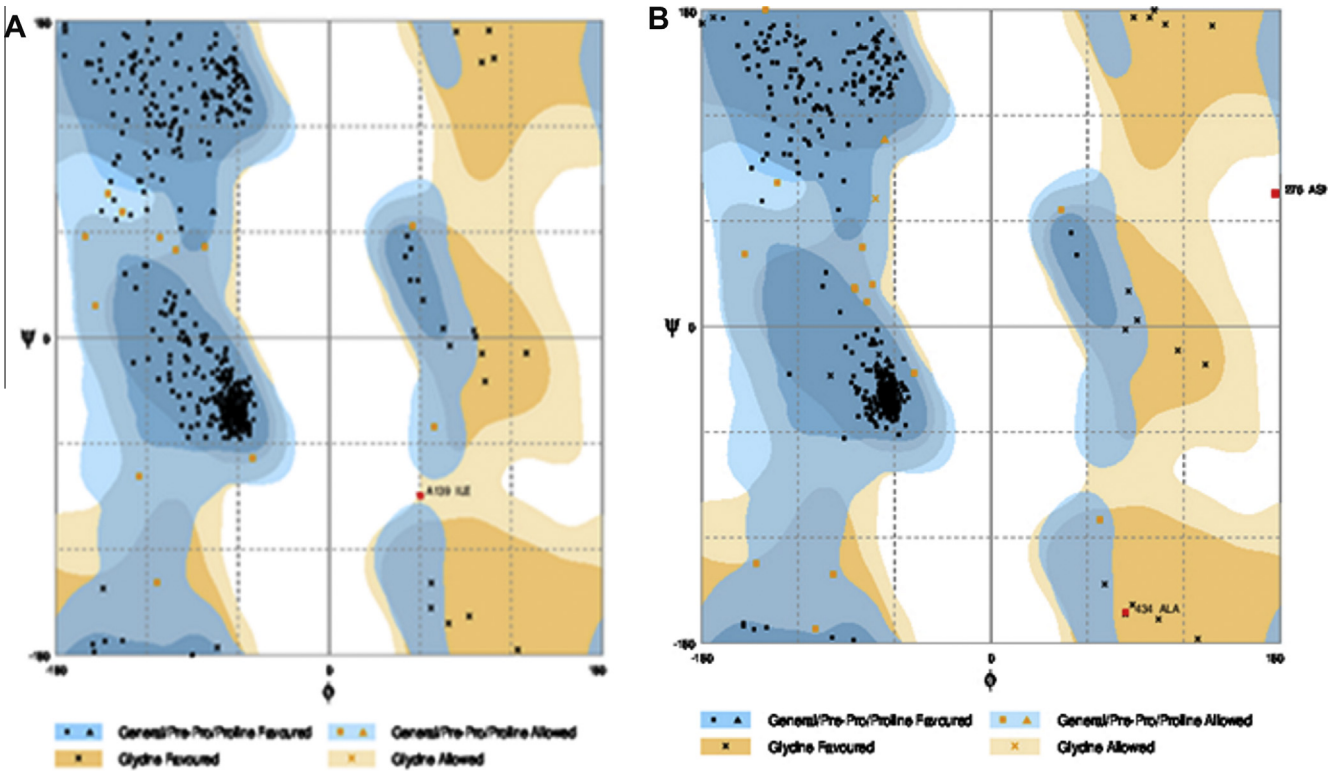

Figure 4 (A) Ramachandran plot of 4K0F and (B) Ramachandran plot of modeled structure.

15 g 2-α-hydroxy phenyl/ethyl-N1-methyl benzimidazole was added slowly with occasional stirring. This mixture was then placed in microwave irradiation at 250 W for 10 min. Excess thionyl chloride was recovered under vacuum on a water bath. To the residue dry dioxane was added and stirred for 30 min. Dioxane was recovered under vacuum to get the final product.

*2.1.6. Synthesis of N-(chloromethyl)-2,4-difluoroaniline (**8**)*

This was done using 2,4-difluroaniline (3 mmol) **7** and dichloromethane (6 mmol) in H_2O (1 ml) using microwave irradiation at 150 °C (power set point 150 W; ramp time 1 min; hold time 20 min). After cooling, the mixture was diluted with water (500 ml), neutralized with $NaHCO_3$, and fractionally distilled at atmospheric pressure through an efficient column, discarding the first fraction and then collecting the pure final product (Marzaro et al., 2009).

*2.1.7. Synthesis of N-((1H-1, 2, 4-triazol-1-yl) methyl)-2,4-difluoroaniline (**9**)*

1,2,4-triazole (10 mmol), NaOH (40 mmol), 2,4-difluro aniline derivative and DMF (10 ml) were stirred on magnetic stirrer for 20 min and then placed in microwave oven for 10 min. The reaction mixture was poured into 50 ml water to obtain the solid precipitate.

*2.1.8. Synthesis of N-(2,4-difluorophenyl)-N-(1-(1,6-dimethyl-1H-benzo[d]imidazol-2-yl)ethyl)-1H-1,2,4-triazol-1-amine (**10a–l**)*

In a typical experiment, 1 g (0.005 mol) of 2-chloromethyl/ethyl/phenyl-N^1-methylbenzimidazole, 1.47 g (0.005 mol) of N-(1H-1,2,4-triazol-1-yl)methyl)-2,4-difluoroaniline were separately dissolved in dry dioxane and mixed in an round bottom flask. To this reaction mixture, 0.76 ml (0.005 mol) of triethylamine was added and the reaction mixture was refluxed for 30 min. The reaction was monitored by TLC. The reaction mixture was then poured into ice cold water and the precipitate was collected by suction and drying. The solid was recrystallized from acetone.

2.2. Compound code

*2.2.1. **10a**: 2.2,4-difluoro-N-((1-methyl-1H-benzo[d]imidazol-2-yl)methyl)-N-(1H-1,2,4-triazol-1-yl) benzenamine*

Compound **10a** was obtained as a white solid (Yield: 76.35%; MP: 114–116; IR (KBr): 3301 (C—N), 2365 (C—H), 1604 (C═N), 1584 (N—N), 1556(C═C), (C—F) 975 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 3.34 (s, 3H, N—CH_3), 4.39 (s, 2H, Ph—CH_2), 6.45–6.51 (m, 3H, Ar—H), 7.30–7.45 (m, 4H, Ar—H of Benzim), 8.42 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 340.12, 325.101, 272, 145. Anal. Calcd. for $C_{17}H_{14}F_2N_6$ (340.33): C, 60.00; H, 4.15; F, 11.16; N, 24.69. Found: C, 60.07; H, 4.21; F, 11.26; N, 24.75.

*2.2.2. **10b**: 2, 4-difluoro-N-(1-(1-methyl-1H-benzo[d]imidazol-2-yl) ethyl)-N-(1H-1,2,4-triazol-1-yl) benzenamine*

Compound **10b** was obtained as a white solid, Yield: 68; mp: 121–123; IR (KBr): 3300 (C—N), 2361 (C—H), 1602 (C═N), 1583 (N—N), 1551 (C═C), (C—F) 972 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 1.49 (d, 1H, —CH—N), 3.18 (s, 3H, N—CH_3), 4.24 (q, 3H, —HC—CH_3), 6.43–6.51 (m, 3H,

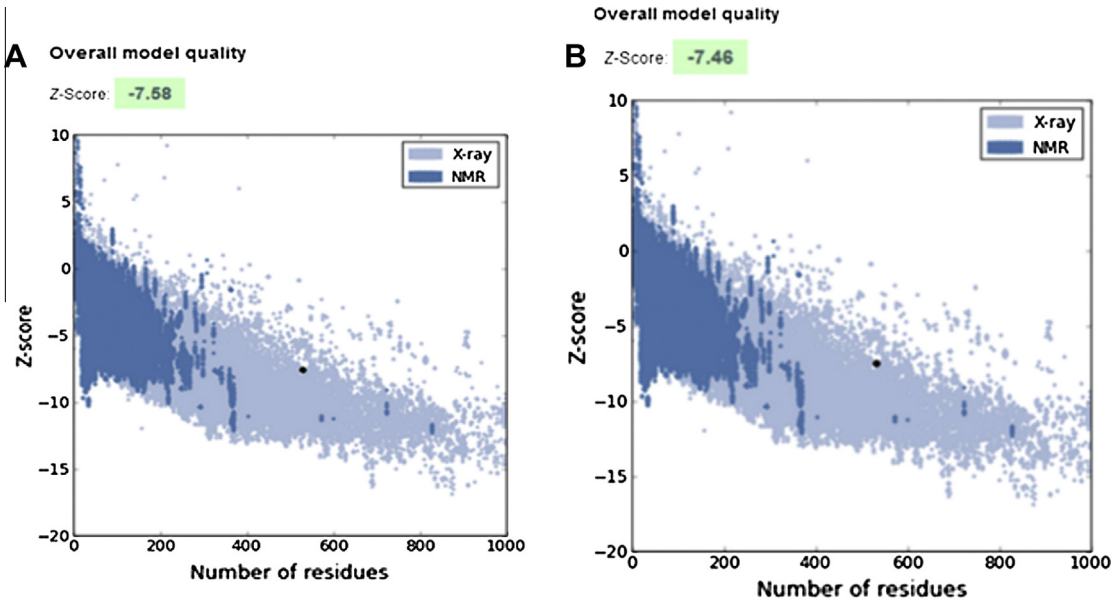

Figure 5 (A) Plot of *Z*-score of 4K0F and (B) plot of *Z*-score of modeled structure.

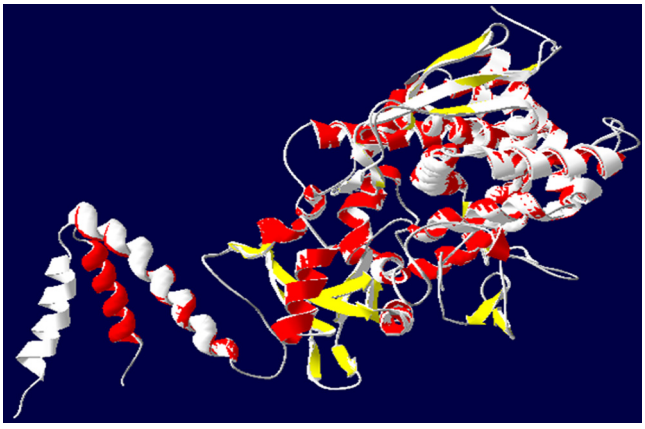

Figure 6 RMSD between modeled structure and template of 0.38 Å. Red color indicates modeled structure of lanosterol 14 alpha demethylase, and white indicates template structure K0F.

Ar—H), 7.26–7.30 (d, 3H, Ar—H of Benzim), 8.02 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 354.1, 339.117, 286.116, 195.048, 159.092. Anal. Calcd. for $C_{18}H_{16}F_2N_6$ (354.1): C, 61.01; H, 4.55; F, 10.72; N, 23.72. Found: C, 61.05; H, 4.50; F, 10.62; N, 23.

2.2.3. ***10c****: N-(2,4-difluorophenyl)-N-((1-methyl-1H-benzo[d] imidazol-2-yl)(phenyl)methyl)-1H-1,2,4-triazol-1-amine*

Compound **10c** was obtained as a white solid, Yield: 71%; mp: 158–160; IR (KBr): 3301 (C—N), 2365 (C—H), 1604 (C═N), 1584 (N—N), 1556 (C═C), (C—F) 975 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 3.62 (s, 3H, N—CH_3), 4.22 (s, 5H, Ar—H), 6.39–6.46 (m, 3H, Ar—H), 7.12–7.22 (d, 1H, —C—H), 7.35–7.47 (d, 3H, Ar—H of Benzim), 8.41 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 401.133, 221.108, 303.136. Anal. Calcd. For $C_{23}H_{18}F_2N_6$ (416.2): C, 66.34; H, 4.36; F, 9.12; N, 20.18. Found: C, 66.24; H, 4.32; F, 9.18; N, 20.12.

2.2.4. ***10d****: 2,4-difluoro-N-((1-ethyl-1H-benzo[d]imidazol-2-yl) methyl)-N-(1H-1,2,4-triazol-1-yl) benzenamine*

Compound **10d** was obtained as a white solid (Yield: 73.10%; mp: 132–134; IR (KBr): 3307 (C—N), 2361 (C—H), 1604 (C═N), 1580 (N—N), 1554(C═C), (C—F) 971 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 3.32 (s, 3H, N-CH_3), 4.36 (s, 2H, Ph-CH_2), 6.41–6.48 (m, 3H, Ar—H),7.14–7.32 (m, 4H, Ar—H of Benzim), 8.58 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 325, 286, 145. Anal. Calcd. for $C_{18}H_{16}F_2N_6$ (354): C, 61.01; H, 4.55; F, 10.72; N, 23.72. Found: C, 62.03; H, 4.32; F, 10.22; N, 23.54.

2.2.5. ***10e****: 2, 4-difluoro-N-(1-(1-ethyl-1H-benzo[d]imidazol-2-yl) ethyl)-N-(1H-1, 2, 4-triazol-1-yl) benzenamine*

Compound **10e** was obtained as a white solid, Yield: 75%; mp: 119–121; IR (KBr): 3306 (C—N), 2364 (C—H), 1602 (C═N), 1574 (N—N), 1560 (C═C), (C—F) 965 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 1.32 (d, 1H, —CH—N), 3.14 (s, 3H, N—CH_3), 4.13 (q, 3H, —HC—CH_3), 6.33–6.48 (m, 3H, Ar—H), 7.20–7.42 (d, 3H, Ar—H of Benzim), 7.94 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 339.117, 300, 195.048, 173. Anal. Calcd. for $C_{19}H_{18}F_2N_6$ (368): C, 61.95; H, 4.93; F, 10.31; N, 22.81. Found: C, 61.24; H, 4.38; F, 10.74; N, 23.02.

2.2.6. ***10f****: N-(2,4-difluorophenyl)-N-((1-ethyl-1H-benzo[d] imidazol-2-yl)(phenyl)methyl)-1H-1,2,4-triazol-1-amine*

Compound **10f** was obtained as a white solid, Yield: 69%; mp: 189–191; IR (KBr): 3288 (C—N), 2345 (C—H), 1615 (C═N), 1571 (N—N), 1545 (C═C), (C—F) 975 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 3.68 (s, 3H, N—CH_3), 4.24 (s, 5H, Ar—H), 6.43–6.51 (m, 3H, Ar—H), 7.26–7.30 (d, 1H, —C—H), 7.26–7.30 (d, 3H, Ar—H of Benzim), 8.02 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 401.133, 362, 235. Anal. Calcd. For $C_{24}H_{20}F_2N_6$ (430): C, 66.97; H, 4.68; F, 8.83; N, 19.52. Found: C, 66.81; H, 4.12; F, 9.32; N, 20.02.

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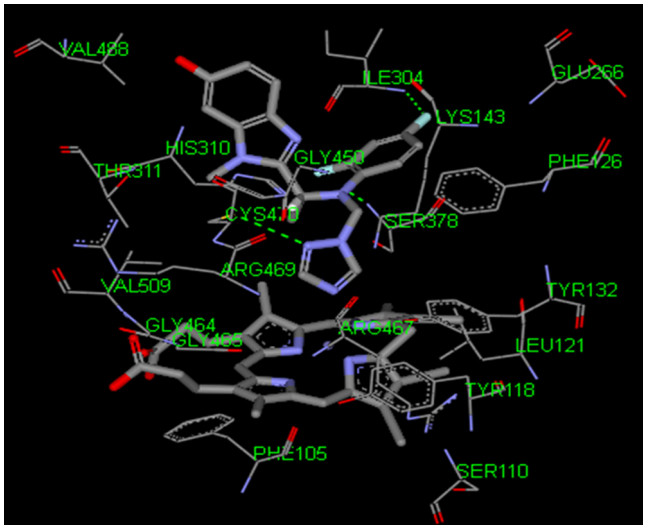


Figure 7 Binding mode of compound **10e** in active site of modeled CYP51 of *Candida albicans*.

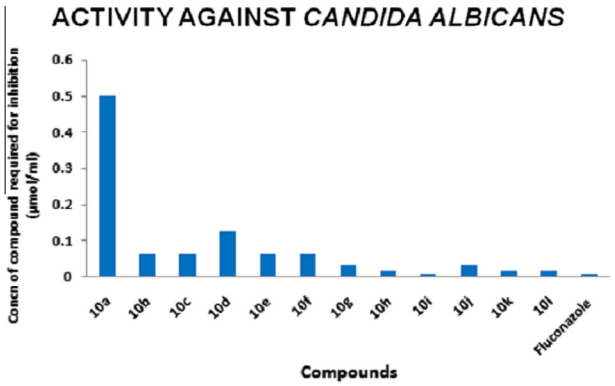


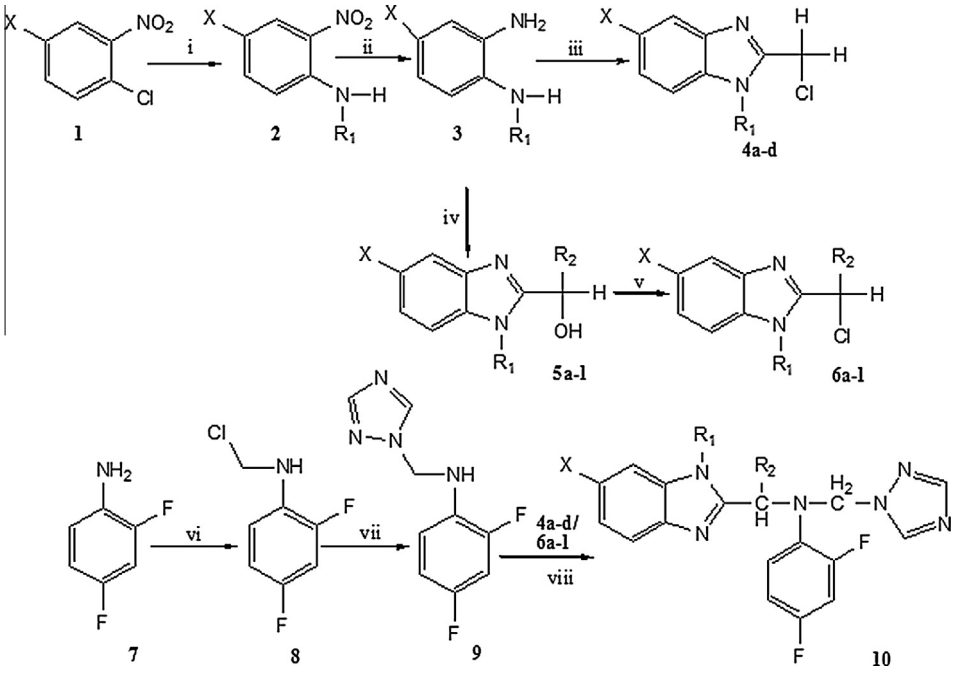
Figure 8 In vitro antifungal activity against *Candida albicans*.

2.2.7. ***10g****: N-(6-chloro-1-methyl-1H-benzo[d]imidazol-2-yl) methyl)-2,4-difluoro-N-(1H-1,2,4-triazol-1-yl) benzenamine*

Compound **10g** was obtained as a white solid, Yield: 64%; mp: 185–187; IR (KBr): 3315 (C—N), 2355 (C—H), 1508 (C═N), 1564 (N—N), 1566 (C═C), (C—F) 970, (C—Cl) 789 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 3.10 (s, 3H, N—CH_3), 4.37 (s, 2H, Ph—CH_2), 6.40–6.58 (m, 3H, Ar—H), 7.20–7.50 (d, 3H, Ar—H of Benzim), 8.15 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 374.8, 359.062, 306.061, 195.048, 179.038. Anal. Calcd. for $C_{17}H_{13}ClF_2N_6$ (374.8): C, 54.48; H, 3.50; Cl, 9.46; F, 10.14; N, 22.42, Found: C, 54.39; H, 3.58; Cl, 9.41; F, 10.11; N, 22.16.

2.2.8. ***10h****: N-(1-(6-chloro-1-methyl-1H-benzo[d]imidazol-2-yl) ethyl)-2,4-difluoro-N-(1H-1,2,4-triazol-1-yl) benzenamine*

Compound **10h** was obtained as a white solid, Yield: 58 %; mp: 144–146; IR (KBr): 3301 (C—N), 2365 (C—H), 1604 (C═N), 1584 (N—N), 1556 (C═C), (C—F) 975, (C—Cl) 789 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 1.49 (d, 1H, —CH—N), 3.18 (s, 3H, N—CH_3), 4.24 (q, 3H, —HC—CH_3), 6.43–6.51 (m, 3H, Ar—H), 7.26–7.30 (d, 3H, Ar—H of Benzim), 8.02 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 370.1, 355.112, 302.11, 195.048. Anal. Calcd. For $C_{18}H_{15}ClF_2N_6$ (388): C, 55.60; H, 3.89; Cl, 9.12; F, 9.77; N, 21.62. Found: C, 55.40; H, 3.81; Cl, 9.08; F, 9.17; N, 21.42.



Scheme 1 Synthesis of tertiary amine type of substituted benzimidazole derivatives. Compound 10: a: X = H, R = H; b: X = OH, R = H; c: X = Cl, R = H; d: X = H, R = CH_3; e: X = OH, R = CH3; f: X = Cl, R = CH3; g: X = H, R = Phenyl; h: X = OH, R = Phenyl; i: X = Cl, R = Phenyl. Reagents and conditions: (i) K_2CO_3, DMF, methylamine; (ii) ammonium chloride, zinc dust; (iii) mono chloro acetic acid, methanol, reflux; (iv) lactic acid/mandelic acid, methanol, reflux; (v) thionyl chloride; (vi) ethanol, KOH, dichloromethane, stir; (vii) 1H, 2,4-triazole, K_2CO_3, toluene; (viii) triethylamine.

*2.2.9. **10i**: N-((6-chloro-1-methyl-1H-benzo[d]imidazol-2-yl)(phenyl)methyl)-N-(2,4-difluorophenyl)-1H-1,2,4-triazol-1-amine*

Compound **10i** was obtained as a white solid, Yield: 63 %; mp: 157–159; IR (KBr): 3280 (C—N), 2367 (C—H), 1607 (C═N), 1584 (N—N), 1536 (C═C), (C—F) 972, (C—Cl) 788 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 3.68 (s, 3H, N—CH_3), 4.24 (s, 5H, Ar—H), 6.43–6.51 (m, 3H, Ar—H), 7.26–7.30 (d, 1H, —C—H), 7.26–7.30 (d, 3H, Ar—H of Benzim), 8.02 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 401.133, 221.108, 303.136. Anal. Calcd. For $C_{23}H_{17}ClF_2N_6$ (450.1): C, 61.27; H, 3.80; Cl, 7.86; F, 8.43; N, 18.64. Found: C, 61.24; H, 3.76; Cl, 7.26; F, 8.33; N, 18.61.

*2.2.10. **10j**: N-(6-chloro-1-ethyl-1H-benzo[d]imidazol-2-yl)methyl)-2,4-difluoro-N-(1H-1,2,4-triazol-1-yl) benzenamine*

Compound **10j** was obtained as a white solid, Yield: 64%; mp: 185–187; IR (KBr): 3312 (C—N), 2341 (C—H), 1513 (C═N), 1571 (N—N), 1560 (C═C), (C—F) 979, (C—Cl) 765 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 3.18 (s, 3H, N—CH_3), 4.21 (s, 2H, Ph—CH_2), 6.54–6.79 (m, 3H, Ar—H), 7.30–7.64 (d, 3H, Ar—H of Benzim), 8.24 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 359, 320, 195.048, 275. Anal. Calcd. for $C_{17}H_{13}ClF_2N_6$ (388): C, 55.60; H, 3.89; Cl, 9.12; F, 9.77; N, 21.62. Found: C, 55.45; H, 3.74; Cl, 9; F, 9.69; N, 21.29.

*2.2.11. **10k**: N-(1-(6-chloro-1-ethyl-1H-benzo[d]imidazol-2-yl)ethyl)-2,4-difluoro-N-(1H-1,2,4-triazol-1-yl) benzenamine*

Compound **10k** was obtained as a white solid, Yield: 58 %; mp: 144–146; IR (KBr): 3306 (C—N), 2358 (C—H), 1604 (C═N), 1586 (N—N), 1552 (C═C), (C—F) 970, (C—Cl) 784 cm^{-1}; ^1H NMR (300 MHz, CDCl3) δ ppm: 1.42 (d, 1H, —CH—N), 3.12 (s, 3H, N—CH_3), 4.14 (q, 3H, —HC—CH_3), 6.33–6.48 (m, 3H, Ar—H), 7.48–7.65 (d, 3H, Ar—H of Benzim), 8.42 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 373, 334, 289. Anal. Calcd. For $C_{19}H_{17}ClF_2N_6$ (402): C, 56.65; H, 4.25; Cl, 8.80; F, 9.43; N, 20.86. Found: C, 55.60; H, 4.12; Cl, 9.04; F, 9.25; N, 21.14.

*2.2.12. **10l**: N-((6-chloro-1-ethyl-1H-benzo[d]imidazol-2-yl)(phenyl)methyl)-N-(2,4-difluorophenyl)-1H-1,2,4-triazol-1-amine*

Compound **10l** was obtained as a white solid, Yield: 63%; mp: 157–159; IR (KBr): 3310 (C—N), 2362 (C—H), 1600 (C═N), 1581 (N—N), 1532 (C═C), (C—F) 970, (C—Cl) 780 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 3.61 (s, 3H, N—CH_3), 4.17(s, 5H, Ar—H), 6.41–6.54 (m, 3H, Ar—H), 7.20–7.28 (d, 1H, —C—H), 7.41–7.58 (d, 3H, Ar—H of Benzim), 8.12 (s, 1H, N═CH—N—CH of 1,2,4-Triazole); ESI–MS *m/z*: 435, 396,351. Anal. Calcd. For $C_{24}H_{19}ClF_2N_6$ (464): C, 62.00; H, 4.12; Cl, 7.63; F, 8.17; N, 18.08. Found: C, 61.91; H, 3.88; Cl, 7.62; F, 8.22; N, 18.01.

2.3. Invitro antifungal activity

The stock solution of (1 µmole/ml) compounds (equimolar mixture) was prepared in DMSO and water. To each tube containing sterilized sabouraud's liquid medium (2 ml), 2 ml of drug solution was added. Each tube was inoculated with the microorganism and was kept at 30 °C for 14 days.

The solutions were first tested at the concentration of 0.5 µmole/ml. The sets, which are found active at this concentration, were again tested at concentration of 0.25 µmole/ml. The groups, which were found active, were subjected to serial dilutions. The serial dilutions were made to obtain concentrations 0.5, 0.25, 0.125, 0.0625, 0.0314, 0.0152 and 0.0076 µmole/ml. Positive control tubes (organism + broth + DMSO) and negative control tubes (broth + drug) were also prepared. Each tube was inoculated with the microorganism.

All the tubes were incubated at 30 °C for 14 days. The readings were taken as expressed as (−) if inhibition of growth is seen and (+) if inhibition of growth is not seen Table 3.

2.4. Invivo antifungal study

2.4.1. Animals

Male Wistar rats (8 weeks; approximately 200 g) were used in this study. Animals were infected intraperitoneal (IP) with *C. albicans* on day 0 (2×105 CFU per mouse); this was followed by IP treatment with test sample. Treatment was given once daily for 4 days starting from day 4 after infection.

2.4.2. Survival study

Survival of the rats was observed until day 45 after infection. For comparison, the survival of rats treated with one of the antifungal agents alone or that received no treatment (untreated controls) was also observed Tables 4A and 4B.

2.4.3. Kidney burden assay

In parallel, groups of five rats each were sacrificed and the numbers of CFU in the kidneys of these rats were determined at the intervals indicated below. Appropriately diluted kidney homogenates were spread onto the surfaces of SDA plates in triplicate, and the numbers of colonies were counted after incubation for 2 days at 30 °C Table 4C.

2.5. Homology modeling of cytochrome P450 lanosterol 14α-demethylase of C. albicans

The 3D model structure of cytochrome P450 lanosterol 14α-demethylase of *C. albicans* was built using homology modeling. Amino acid sequence of enzyme was obtained from the Universal Protein Resource (http://www.uniprot.org/) (Accession Code: P10613), and sequence homologous was obtained from Protein Data Bank (PDB) using Blast search. In literature (Monk et al., 2014), the structure of cytochrome P450 lanosterol 14α-demethylase was developed homologically using crystal structure of lanosterol 14α-demethylase from *Saccharomyces cerevisiae* YJM 789 as template (531 amino acid residues). Based on the result of blast search, we used the crystal structure of *Saccharomyces cerevisiae* YJM 789 lanosterol 14α-demethylase (CYP51) with intact transmembrane domain bound to itraconazole as a template for homology modeling (PDB ID. 4K0F, RESOLUTION. 2.19 Å). The sequence alignment of 4K0F was done using discovery studio showed identity 55.9% and similarity 70.2% with our target sequence Fig. 2. Further, the homology modeled protein Fig. 3 energy minimization and loop refinement are carried out by applying CHARMm force fields and smart minimization algorithms followed by conjugate gradient algorithms

until convergence gradient was satisfied. These procedures were performed by discovery studio (Lovell et al., 2003).

Geometric evaluations of the modeled 3D structure were performed using Rampage Fig. 4A and B. The Ramachandran plot of our model showed that 96.6% of residues were in the favor, 3% allowed regions, and 0.4% was in the outlier region as compared to 4K0F template (98%, 2%, and 0%). The plot statistics are presented in Table 1.

Validation was carried out using ProSA to obtain the *Z*-score value for the comparison of compatibility Fig. 5. The *Z*-score plot showed spots of *Z* score values of proteins determined by NMR (represented in dark blue color) and by X ray (represented in light blue color). The two black dots represent *Z*-scores of our model (−7.46) and template (−7.58). These scores indicate the overall quality of the modeled 3D structure of lanosterol 14-alpha demethylase of *C. albicans*. RMSD (0.38 Å) was calculated between the main chain atom of model and template. It indicated close homology. This ensured the reliability of the model. Superimposition between target and template structure was done using SPDBV Fig. 6.

3. Results and discussion

3.1. Synthesis

The targeted compounds were synthesized by a seven-step reaction process (Scheme 1). The 4-substituted N1-alkyl-2-nitroaniline **2** was synthesized by using 4-substituted 2-chloronitrobenzene **1**, anhydrous potassium carbonate, alkylamine and dimethyl formamide (DMF) as solvent in microwave. The nitro group of phenyl ring **2** was further reduced to amino group in presence of ammonium chloride and zinc dust to give **3**. Further this synthesized **3a–d** and mono chloro acetic/lactic/mandelic acid dissolved in methanol, HCl and reflux in microwave to give **4a–d** and **5a–h**. Then activation of carboxylic functional group of **5a–h** was carried out by using thionyl chloride and catalytic amount of DMF to give **6a–h**. 2, 4-Difluroaniline and dichloromethane were dissolved in ethanol and stirred on Rota mental at 750 C to give **8**. Further **8** reacted with 1H, 2, 4-triazole in presence of NaOH and DMF to give **9**. The target compounds **10a–i** were obtained by reacting **9** and **4a–d**, **6a–h**, in the presence of triethylamine.

3.2. Docking

The ligand fit method was performed to study and predict the binding mode of newly synthesized compounds with the target enzyme (homology modeled) cytochrome P450 lanosterol 14-α-demethylase of *C. albicans*. All compounds showed binding in the active site of the enzyme. The 1, 2, 4-triazole ring (compounds **10a–10l**) is positioned almost perpendicular to the porphyrin plane, with a ring nitrogen (N-4) atom coordinated to the heme iron (Fig. 7). The distance between nitrogen of azole ring in all compounds and heme ring was measured and found in the range of 2.16–3.19 and was comparable with that found in the crystal structure of human lanosterol 14α-demethylase complexed with azole inhibitors Table 2. The 1, 2, 4-triazole ring nitrogen atom (N2) forms hydrogen bonding with amino acid CYS 470. The tertiary amine group showed hydrogen bonding with SER378. In most of the compounds, dihalophenyl ring occupied the same hydrophobic region above the heme ring and showed good Vander Waals interaction with heme and amino acids ILE304, LYS143, and GLY450, whereas substituted benzimidazole ring of most of compounds located near hydrophobic region by making good Vander Waals interaction with HIS 310, THR311, VAL488, and MET508. In particular, all hydrophobic substituents find location in a hydrophobic sub-site above the heme ring.

3.3. Biological activity

Antifungal activity of the synthesized compounds was tested against candida albicans spores *in vitro* and *in vivo*. *In vitro* antifungal activity was evaluated using the tube dilution method (Cappucino and Sherman, 1996; Pernaka et al., 2001) (turbidimetric method). The turbidimetric method depends on the inhibition of growth in a microbial culture of uniform solution containing the drug in fluid medium favorable for rapid growth. In this method, minimal inhibitory concentration (MIC) of the antifungal agent was determined. The MIC is the lowest concentration of an antimicrobial agent that inhibits the test organism. The growth in the tube was observed visually for turbidity and inhibition was determined by the absence of growth. In the present study, *C. albicans* (ATCC10232) was used to investigate the activity. Fluconazole and dimethyl sulphoxide were used as standard and solvent respectively.

The antifungal activity clearly indicated that **10i** has a good antifungal activity as compared with the other twelve compounds at 0.0075 μmole/ml which is equivalent to fluconazole activity as far as the *in vitro* results are concerned. The activity decreased with the increasing alkyl length of N1 of benzimidazole (methyl to ethyl). This was proven with compounds **10i** and **10l** which showed MICs of 0.0075 and 0.015 μmoles/ml respectively Fig. 8.

C. albicans induced candidiasis in rats was used to investigate the *in vivo* antifungal activity of test samples. It is also proposed that kidney is one of the prime organs affected by fungal infection in *in vivo* models. In systemic candidiasis, kidney is the major site of multiplication of *C. albicans*. The animals were infected with *C. albicans* and fungal load was characterized on the basis of kidney burden of *C. albicans*. Kidney homogenate was serially diluted to get proper results to count colonies of *C. albicans*. Colony forming units (CFU) in kidney homogenate of animals treated with standard drug and test samples were significantly less ($P < 0.001$) as compared to vehicle treated animals. All test samples were effective at 0.26 and 0.42 mg/kg. Compound **10i** showed the most superior activity. CFU in kidney homogenate of compound **10i** treated animals was significantly less ($P < 0.001$) as compared to other treatments. The results confirmed that **10i** was more potent as compared with the other compounds. Significantly lesser value of fungal burden in kidney of test sample treated animals confirmed antifungal potential of test samples.

4. Conclusion

In conclusion, a new scaffold has been proposed for potential antifungal activity. Benzimidazole derivative with N1 methyl, C2 as asymmetric center, C5 chlorine substitutions, tertiary amine and 1,2,4-triazole showed good docking score and antifungal activity. This may be due to increase in basicity of

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N1 and hydrophobicity due to phenyl ring on C2 of benzimidazole. Docking scores and antifungal activity of these compounds have been justified by investigating their interaction at the active site of CYP51. The 1,2,4-triazole ring is positioned almost perpendicular to the porphyrin plane, with a ring nitrogen (N-4) atom coordinated to the heme iron and nitrogen atom (N2) forms hydrogen bonding with amino acid CYS 470. The tertiary amine group showed hydrogen bonding with SER378. The structural difference of these compounds from the fluconazole can make them less prone to the development of resistance by pathogenic fungal strain against them. A meaningful SAR has been developed.

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